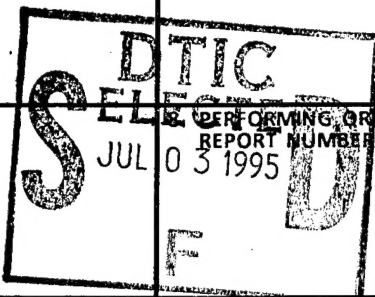


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13. ABSTRACT (Maximum 200 words) Protein-protein and intraprotein interactions were studied in natural, recombinant and synthetic domains of proteins that form aquatic silk. Subsets of natural proteins were found to associate into discrete high molecular mass complexes thought to be the non-covalent precursors to insoluble silk. A recombinant protein mimicking a single "core repeat" from a 1000-kDa silk protein reversibly formed two intramolecular disulfide bonds; however, more than one conformation existed. A synthetic peptide encompassing all four cysteines also formed intramolecular disulfide bonds. The results obtained suggested new steps in the fiber formation pathway including properties of the recombinant protein that may render it useful as a degradable biomolecular material. DTIC QUALITY INSPECTED 5				
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8. STATEMENT OF PROBLEM

The aim of this project was to study the structure, synthesis, intraprotein and protein-protein interactions of a family of secreted silk proteins that are synthesized in larval salivary glands of the midge, *Chironomus tentans*. These silk proteins are mainly composed tandem copies of novel repeated amino acid sequences and assemble *in vivo* into complexes that form insoluble silk fibers. Repeated domains from silk proteins made provide unique and useful protein-based polymers.

9. SUMMARY OF IMPORTANT RESULTS

(Superscripts refer to PUBLICATIONS listed in section 10.)

A. *Chironomus* Silk is Unique

Why study the silk of a lesser known Dipteran, like *Chironomus*, when silk is spun by more well known arachnids and several insects orders? First, while most known silks have similar amino acid compositions, *Chironomus* silk is the only one with significant amounts of cysteine (Cys)⁵. Second, the molecular biological data base for *Chironomus* silk proteins (including but not limited to the chromosomal location, organization and developmentally regulated expression of thirteen silk protein genes and their encoded primary amino acid sequences) exceeds that for any other organism¹. Third, intermediate and large size classes of silk proteins are composed of 50-130 tandem copies of novel amino acid sequences punctuated by conserved Cys-containing motifs¹. The uniqueness of these proteins provides an opportunity to study a natural biopolymer with potential application of biomolecular materials.

B. Silk Protein Interactions *in Vivo*

Insoluble *Chironomus* silk is spun from a reservoir of soluble silk proteins in the salivary gland lumen. Optical characterization of luminal proteins indicates their ability to form nematic liquid crystals that likely assemble from supermolecular aggregates^{4,7}. Presumably, these "aggregates" form the networks of multi-stranded beaded fibers which we previously demonstrated can be disassembled and reassembled *in vitro*. Development of a suitable system for non-denaturing agarose gel electrophoresis⁹ provided initial evidence for the existence of such "aggregates" which were subsequently identified¹² as discrete silk protein complexes: complex C1a nominally contains sp1a + sp185 + sp140; C1b contains sp1b + sp185. These complexes are primarily stabilized by non-covalent interactions, though a small percentage of molecules exhibit intermolecular disulfide bonds. Other proteins (sp40, sp17 and sp12) co-sediment with C1a and C1b and may also be part of these complexes which, in turn, aggregate into multimeric oligomers. Isolated complexes assemble *in vitro* into multi-stranded beaded fibers¹², the precursors to silk threads.

C. Synthetic and Recombinant Silk Protein Interactions *in Vitro*

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The paucity and heterogeneity of natural silk proteins renders them unsuitable reagents for in-depth structural studies. To overcome this limitation, we synthesized peptides and recombinant proteins as model substrates for detailed biophysical and biochemical studies. Since spIs form the fibrous backbone of silk, we chose to study their core repeats. The "constant regions" of these repeats has four Cys residues that in synthetic peptides⁶ and recombinant proteins^{3,8,10} reversibly form two disulfide bonds. The major focus of this work was rCAS, a recombinant Constant And Subrepeat (rCAS) protein modeled after a core repeat from spIa³.

Characterization of rCAS included polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, quantitative amino acid composition, quantitative sulfhydryl assays, matrix-assisted laser desorption/time-of-flight and electrospray ionization mass spectrometry and mapping disulfide-containing tryptic peptides¹⁰. As purified, there are three rCAS conformers that differ in their Cys-pairing. Denatured and reduced rCAS was subjected to refolding at pH 8 and 10 with O₂- and DTT^{ox}-mediated formation of disulfide bonds. The kinetics of refolding was monitored at different times by HPLC. While the rates of the reactions varied, in all instances, reduced rCAS passed through one disulfide-containing intermediates on the way to reforming two disulfide bonds; however, heterogeneity of Cys pairs persisted suggesting that rCAS may have more than one stable structure. The occasional appearance of rCAS dimers and trimers prompted us to investigate aggregation in solution by analytical equilibrium ultracentrifugation. At protein concentrations ranging from 0.25-1.0 mg/ml in 100 mM Na⁺, rCAS is a monomer¹⁰.

Using *in vitro* mutagenesis, a set of 20 genes was made that encompass all single, double, triple and quadruple Cys→Ala substitutions. Most also include a Phe→Trp substitution that provides an intrinsic fluorochrome at one specified site. We subcloned 19/20 mutant genes into expression vector pET3a and obtained inducible synthesis of all recombinant proteins. Selected variants are being purified and subjected to biochemical verification of their encoded amino acid substitutions by amino acid composition, sulfhydryl counting gels, mass spectrometry, and UV absorption and fluorescence spectroscopies. Preliminary data are exciting. For example, one Cys→Ala substitution alters reformation of disulfide bonds⁸ and the quantum yield of fluorescence for QUAD (Cys_{9,21,23,79}→Ala) is similar to free Trp whereas F16W exhibits a decreased yield that can be increased by denaturation. This suggests that while the sole Trp in F16W is buried, in QUAD, which lacks Cys (hence no disulfide bonds), it is exposed to the solvent. These results have set the stage for a detailed structural and thermodynamic comparison of these proteins.

D. Related Proteins

Several collaborations have enabled us to examine other Cys-containing proteins and gain experience with biophysical methods. For example, the homologue of *C. tentans* sp185 (motif Cys-X-Cys-X-Cys every 22-26 residues) has been identified in two other species of *Chironomus*². Cys-rich chorion (egg-shell) shell proteins in two Lepidopteran have been examined¹¹. Native, synthetic and recombinant proteins have been examined by a variety of spectroscopic methods. Most notably we have acquired the capacity to conduct FTIR of proteins

in solution; this offers advantages over the typical acquisition of spectra in KBr pellets. A spectral library of known proteins has been assembled to provide basis spectra from which quantitative estimates of the percentages of secondary structures can be made for unknown proteins.

10. PUBLICATIONS AND TECHNICAL REPORTS

1. Case, S.T. and Wieslander, L. Secretory proteins of *Chironomus* salivary glands: Structural motifs and assembly characteristics of a novel biopolymer. *Results and Problems in Cell Differentiation*. Vol. 19, *Structure, Cellular Synthesis and Assembly of Biopolymers*. (S.T. Case, ed.), pp. 187-226, Springer-Verlag, Berlin Heidelberg (1992).
2. Brumley, L.L. Bogachev, S., Kolesnikov, N.N., Waite, J.H. and Case, S.T. Divergence and conservation of epitopes in intermediate size secretory proteins from three species of *Chironomus*. *Comp. Biochem. Physiol.* 104B:731-738 (1993).
3. Smith, S.V. and Case, S.T. Over-expression of a core repeat from an insect silk protein that forms intramolecular disulfide bonds. *Biomolecular Materials*, Vol. 292, *Materials Research Society Symposium Proceedings* (C. Viney, S.T. Case, and J.H. Waite, eds.), pp.93-98, Materials Research Society, Pittsburgh (1993).
4. Viney, C. Huber, A., Dunaway, D. Case, S.T., and Kaplan, D.L. Processing biological polymer solutions: Kinetic aspects of microstructural control. *Biomolecular Materials*, Vol. 292, *Materials Research Society Symposium Proceedings* (C. Viney, S.T. Case, and J.H. Waite, eds.), pp.211-217, Materials Research Society, Pittsburgh (1993).
5. Case, S.T., Powers, J., Hamilton, R. and Burton, M.J. Silk and silk proteins from two aquatic insects. In *Silk Polymers. Materials Science and Biotechnology*. Vol. 544, *American Chemical Society Symposium Series*, (D. Kaplan, W.W. Adams. B. Farmer and C. Viney, eds.), pp. 80-90, American Chemical Society, Washington (1994).
6. Case, S.T. and Smith, S.V. Synthetic and recombinant domains from a midge's giant silk protein: role for disulfide bonds. In *Silk Polymers. Materials Science and Biotechnology*. Vol. 544, *American Chemical Society Symposium Series*, (D. Kaplan, W.W. Adams. B. Farmer and C. Viney, eds.), pp. 91-97, American Chemical Society, Washington (1994).
7. Viney, C. Huber, Dunaway, D.L., Kerkam, Case, S.T. Optical characterization of silk secretions and fibers. In *Silk Polymers: Materials Science and Biotechnology*. Vol. 544, *American Chemical Society Symposium Series*, (D. Kaplan, W.W. Adams. B. Farmer and C. Viney, eds.), pp. 120-136, American Chemical Society, Washington (1994).
8. Case, S.T., Smith, S.V. and Bratton, M.R. Disulfide bonds in recombinant repeat units

from an aquatic insect's silk protein. *Biomolecular Materials by Design*, Vol. 330, *Materials Research Society Symposium Proceedings* (M. Alper, H. Bayley, D. Kaplan, and M. Navia, eds.), pp. 31-36, Materials Research Society, Pittsburgh (1994).

9. Thornton, J.R., Daum, H.A., and Case, S.T. Agarose gel electrophoresis of high molecular mass protein complexes. *BioTechniques* 18:324-327 (1995).
10. Smith, S.V., Correia, J.J., and Case, S.T. Disulfide bonds in a recombinant protein modeled after a core repeat in an aquatic insect's silk protein. *Protein Science* 4: 945-954 (1995).
11. Orfanidou, C.C., Chryssikos, G.D., Kamitsos, E.I., Wellman, S.E., Case, S.T. and Hamodrakas, S.J. Spectroscopic studies of *Manduca sexta* and *Sesamia nonagriodes* chorion protein structure. *Int. J. Biol. Macromol.* (in press, 1995).
12. Case, S.T. and Thornton, J.R. High molecular mass complexes of *Chironomus tentans* His proteins. (manuscript in preparation)

11. PARTICIPATING SCIENTIFIC PERSONNEL

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